ELSEVIER

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Overexpression of NRPS4 leads to increased surface hydrophobicity in *fusarium graminearum*

Frederik Teilfeldt HANSEN[a], *Aida DROCE*[a,b], *Jens Laurids SØRENSEN*[a,b], *Peter FOJAN*[c], *Henriette GIESE*[a,b], *Teis Esben SONDERGAARD*[a,b,*]

[a]*Department of Animal Sciences, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark*
[b]*Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, 9000 Aalborg, Denmark*
[c]*Department of Physics and Nanotechnology, Aalborg University, Skjernvej 4, 9000 Aalborg, Denmark*

ARTICLE INFO

Article history:
Received 28 February 2012
Received in revised form
16 April 2012
Accepted 20 April 2012
Available online 3 May 2012
Corresponding Editor:
Simon V. Avery

Keywords:
Fusarium
Fusarin c
Hydrophobicity
Nonribosomal peptide synthetases
Secondary metabolism

ABSTRACT

The plant pathogen *Fusarium graminearum* is the infamous cause of Fusarium head blight worldwide resulting in significant losses of yield and reduced grain feed quality. It also has the potential to produce a range of small bioactive peptides produced by the non ribosomal peptide synthetases (NRPSs). Most of these are unknown as *F. graminearum* contains 19 NRPS encoding genes, but only three have been assigned products. For the first time, we use deletion and overexpression mutants to investigate the functions and product of *NRPS4* in *F. graminearum*. Deletion of *NRPS4* homologues in *Alternaria brassicicola* and *Cochloibolus heterostrophus* has been shown to result in mutants unable to repel water. In a time study of surface hydrophobicity we observed that water droplets could penetrate 7 d old colonies of the *NRPS4* deletion mutants. Loss in ability to repel water was first observed on 13 d old cultures of the wild type strain, whereas the overexpression strain remained water repellant throughout the 38 d time study. The conidia of both mutants were examined and those of the overexpression mutant showed distinct morphological differences in form of collapsed cells. These observations might suggest that the peptide product of *NRPS4* could be an architectural factor in the cell walls of *Fusarium* or an indirect regulator of hydrophobicity.

Introduction

The nonribosomal peptide synthetases (NRPSs) of filamentous fungi are large multifunctional enzymes which assemble amino acid substrates into small peptides and thereby circumvent the ribosomal system. The NRPSs consist of modules each capable of selecting and acquiring a specific amino acid substrate by its adenylation (A) domain and attaching it to the growing peptide chain through a thiotemplate system (von Döhren, 2004; von Döhren *et al.*, 1999). The modular structure of the NRPS is normally composed of a number of elongation modules consisting of condensation, adenylation and thiolation domains in that order $(C\text{-}A\text{-}T)_n$ (Strieker *et al.*, 2010). These systems are widespread throughout bacteria and fungi and the products often have bioactive properties such as antibiotic, fungicidal or immunosuppressive, which are believed to be employed as weapons to secure the ecological niche of the organism in nature (Calvo *et al.*, 2002; von Döhren, 2004).

* *Corresponding author*. Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, 9000 Aalborg, Denmark. Tel.: +45 9940 8524; fax: +45 9814 1808.
E-mail address: tes@bio.aau.dk
1878-6146/$ – see front matter
doi:10.1016/j.funbio.2012.04.014

The filamentous fungus *Fusarium graminearum* is a major plant pathogen of wheat and barley and causes Fusarium head blight resulting in severe losses and contamination of grains with mycotoxins such as deoxynivalenol (DON) (Gardiner *et al.*, 2009). The sequenced *F. graminearum* has revealed 19 NRPS genes (Hansen *et al.*, 2012; Tobiasen *et al.*, 2007; Varga *et al.*, 2005). Only the products of three are known all of which are iron chelating siderophores. None of the other NRPs have been linked to their respective synthetase gene (Bushley *et al.*, 2008; Tobiasen *et al.*, 2007). Bioinformatic tools exists for the prediction of amino acid substrates of bacterial NRPSs but are not always applicable for fungi as these are more complex and can incorporate a significantly higher number of different substrates (~500) compared to bacteria (Strieker *et al.*, 2010).

The *NRPS4* gene of *F. graminearum* (FGSG_02315) encodes an 844 kDa (7639 amino acids) NRPS (Fig 1) consisting of five modules and is conserved throughout the four sequenced *Fusarium* species, *F. graminearum, F. oxysporum, F. solani*, and *F. verticillioides* (Hansen *et al.*, 2012). It is also present in range of other plant pathogenic filamentous fungi.

The *NRPS4* of *Cochliobolus heterostrophus* is a likely orthologue of *FgNRPS4* and has a similar modular structure. *ChNRPS4* has been reported to be involved in surface hydrophobicity and deletion of *ChNRPS4* led to a loss in ability to repel water (Turgeon *et al.*, 2008). *NRPS2* in *Alternaria brassicicola* is another possible orthologue and here deletion mutants also show a decreased hydrophobicity in addition to abnormal conidia cell wall morphology, decreased sporulation and germination resulting in a reduced infection capability (Kim *et al.*, 2007). *NRPS4* has some similarity to *pes1* from *Aspergillus fumigatus*. This gene has been linked to resistance to oxidative stress and pathogenicity and deletion of the gene impaired both virulence and increased sensitivity to oxidative stress (Reeves *et al.*, 2006).

Here we investigate *NRPS4* from *F. graminearum* by generation of deletion and overexpression mutants and characterization of their phenotypes. Both mutants had altered hydrophobic properties and different extraction methods were employed in the abortive attempt to isolate the NRPS4 peptide. The conidial morphology of the mutants was investigated by atomic force microscopy.

Materials and methods

Generation of NRPS4 overexpression and deletion mutants

For generation of an overexpression plasmid construct a flanking 751 bp fragment upstream of *NRPS4* was amplified using the primers NRPS4o1 and NRPS4o2 (primer sequences are found in Supplementary Table 1). The PCR was performed with the PFU C turbo polymerase (Stratagene, La Jolla, CA, USA) on genomic DNA from *Fusarium graminearum* isolate PH-1 according to the manufacturer's instructions. An additional 733 bp fragment was amplified at the beginning of the gene with the primers NRPS4o3 and NRPS4o4. The PCR fragments were cloned into a linearized pRF-HU2E vector under control of the gpdA promoter by a four fragment cloning step using the USER enzyme™ (New England Biolabs, Ipswich, MA, USA) and verified by colony PCR according to (Frandsen *et al.*, 2008). The same method was used to generate a deletion construct. Here the initial upstream flanking fragment was used in addition to a 569 bp downstream flanking fragment generated using the primers NRPS4a3 and NRPS4a4. The deletion construct was verified by colony PCR with the RF2 and RF4 primers as well as with the RF1 and RF8 primers. Prior to transformation into *Agrobacterium tumefaciens* the fidelity of the constructs were checked by sequencing at Eurofins MWG Operon (Ebersberg, Germany) using the primers RF2 and RF8. The transformed *A. tumefaciens* were proliferated in minimal media (17 mM K_2HPO_4 (pH 7.0), 8 mM NaH_2PO_4, 18 mM NH_4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 90 μM $CaCl_2$, 147 μM $FeSO_4$ and 0.5 % (w/v) glucose) and transferred to IMAS medium (0.04 M MES, 0.2 mM Acetrosyringon, 0.2 % (w/v) glucose, 0.5 % (v/v) glycerol, 0.01 M KH_2PO_4, 0.01 M K_2HPO_4, 2.6 mM NaCl, 2 mM $MgSO_4$, 3.4 mM $CaCl_2$, 9 μM $FeSO_4$ and 3.6 μM $(NH_4)_2$ SO_4). The *F. graminearum* transformation was carried out as previously described (Malz *et al.*, 2005).

Verification of mutants

The mutants were screened by colony PCR for correct insertion, using one primer specific for the *hyg* gene and another corresponding to a flanking region outside the insertion area, and the number of inserts was determined by Southern blotting. Genomic DNA was extracted from the transformed fungi using the DNeasy plant mini kit (Qiagen, Hilden, Germany) and subjected to a restriction digestion using *HindIII* (New England Biolabs). A hybridization probe of 588 bp corresponding to a part of the *hyg* gene of the plasmid vector was amplified by PCR. The digested gDNA was separated on an agarose gel and the blotting and following hybridization were performed according to the manufacturer's instructions (GE Healthcare Life Sciences) using a Hybond N+ membrane. Nucleic acids were visualized using the AlkPhos Direct Labeling and Detection System with CDP-*Star* (GE Healthcare Life Sciences).

Transcription analyses

Reverse Transcriptase (RT) - PCR was performed to determine expression of *NRPS4* in the wild type and mutant strains grown on YES medium for seven days as described in (Sørensen *et al.*, 2012). The fungal tissue was removed from

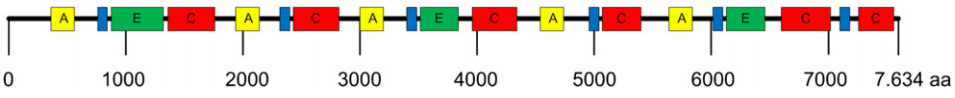

Fig 1 – Domain architecture of the five module NRPS4 of *F. graminearum*. The colors indicate the type of domain: Yellow; adenylation domain, blue; thiolation domain, green; epimerization domain and red; condensation domain.

the medium, lyophilized and pulverized by the addition of a mix of glass beads which were shaken at 2 × 10 seconds intervals in a bead beater. The RNA was then extracted using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The RNA quality was checked by agarose gel electrophoresis and first stand synthesis was performed with random hexamer primers (Eurofins MWG) using the SuperScript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) with the addition of RNaseOUT according to the manufacturer's instructions.

The RT-PCR was performed by 28 cycles of PCR using the DyNAzyme polymerase (Finnzymes, Oy, Finland) according to the manufacturer's instructions. Two housekeeping genes β-tubulin (accession number; FGSG_06611) and transcription elongation factor 1 α (accession number; FGSG_08811) were amplified as controls.

Time study of surface hydrophobicity

Time studies were carried out on wild type PH1, OE*NRPS4* and Δ*NRPS4* mutants. Spores were used to inoculate YES plates and incubated at 25 °C in the dark. Plates of each strain were examined for surface hydrophobicity by the addition of a 50 μL drop of water to individual plates 11 times during the 38 d of the experiment.

Characterization of conidia

To analyze conidia phenotypes of the wild type and the mutant strains by atomic force microscopy (AFM) petri dishes with a glass slide inserted in the bottom (Willco Wells) were incubated with a spore solution. After 30 min. the supernatant was discarded and the spores rinsed with water, and dried under a stream of nitrogen. AFM semi contact mode images were recorded at room temperature on an NT-MDT Ntegra Life AFM microscope and an Olympus cantilever (OMCL 200) with a resonance frequency of 138 kHz and a spring constant of 9N/m.

Metabolite analyses

Nine plugs of 14 days old cultures grown on solid YES medium were extracted with 1.5 mL ethyl acetate-dichloromethane-methanol (3:2:1) according to (Smedsgaard, 1997). The extracts were evaporated to dryness under a stream of nitrogen and dissolved in 500 μL methanol. Five μL extracts were separated on a Gemini C6-Phenyl 3 μm 2 mm i.d. × 100 mm column (Phenomenex, Torrance, CA, USA) on an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) equipped with a diode array detector collecting two ultraviolet–visible (UV–VIS) spectra per second from 200 to 600 nm. An acetonitrile:water gradient was used starting at 0 % acetonitrile increasing to 100 % in 20 min, which was held for 5 min before reverting to 0 % in 3 min. A flow rate of 0.4 mL/min was used and both eluents were buffered with 50 ppm trifluoroacetic acid.

Results

Generation of NRPS4 overexpression and deletion mutants

Vectors for overexpression and deletion of *NRPS4* in *Fusarium graminearum* were generated as described in Fig 2A. Correct integration of the overexpression and deletion vectors into the

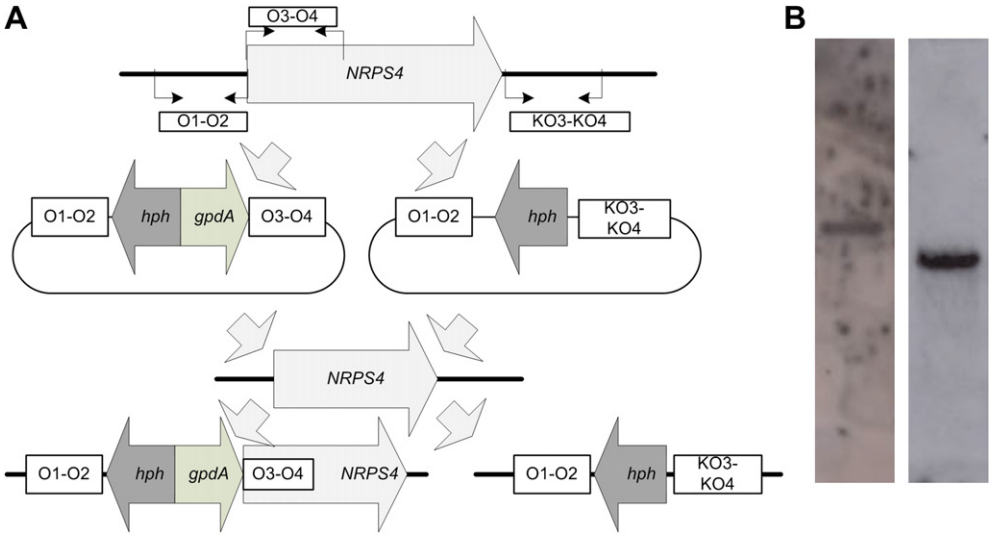

Fig 2 – Construction of deletion and overexpression *NRPS4* mutants. A) USER cloning strategy. Generation of overexpression mutants are obtained by cloning amplified regions O1-O2 and O3-O4 into a p-HU2E vector containing the *hph* gene (hygromycin B phosphotransferase) and the gpdA constitutive promoter. Following *A. tumefaeciens* mediated homologue recombination the resulting OE*NRPS4* strain is shown to the left. To the right is shown the generation of the Δ*NRPS4* using the KO3-KO4 amplicon, replacing the *NRPS4* with the *hph* gene. B) Southern blot of OE*NRPS4* (7800 bp) (left) and the Δ*NRPS4* (3500 bp).

genome had been achieved and Southern blotting showed that only a single copy had been inserted (Fig 2B).

Time study of surface hydrophobicity

Deletion of *NRPS4* homologues in *Alternaria brassicicola* and *Cochloibolus heterostrophus* has been shown to result in mutants unable to repel water. To examine whether deletion of *NRPS4* in *Fusarium graminearum* has a similar effect we set up a time study where water droplets were added to different cultures at day 4, 7, 10, 13, 16, 19, 20, 23, 26, 30, and 38. The results showed that the Δ*NRPS4* mutant displayed a distinct lack of surface hydrophobicity and was unable to repel water when grown on a YES medium shortly after 7 d of growth (Fig 3A). The wild type strain was water repellant at day 10, but not 13, whereas the OE*NRPS4* mutant displayed a constant increased surface hydrophobicity throughout the experiment.

To link the observed differences in hydrophobicity to *NRPS4* we examined transcription of the gene by RT-PCR on 7 d old cultures grown on YES medium (Fig 3B). Expression of *NRPS4* was observed in the wild type strain and OE*NRPS4*, but not in Δ*NRPS4*. This further verifies that the *NRPS4* gene has successfully been deleted in Δ*NRPS4*. The RT-PCR of *NRPS4* resulted also in a more intense band for OE*NRPS4* compared to the wild type, which shows that the expression of the gene is up-regulated in the mutant.

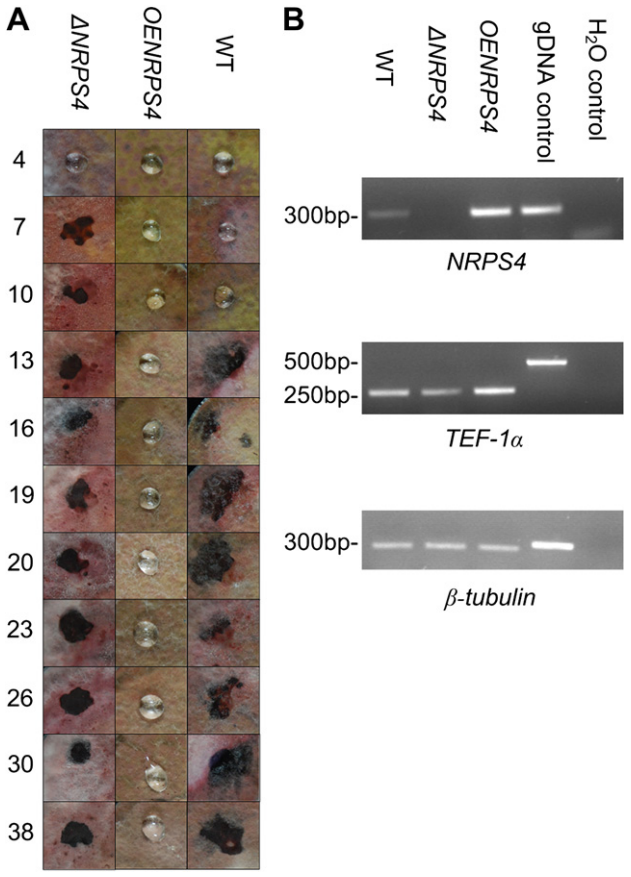

Fig 3 – A) Time study of the surface hydrophobicity of wild type PH1, OE*NRPS4* and Δ*NRPS4*. B) RT-PCR of *NRPS4* with β-tubulin and elongation factor 1α as control of wild type PH1, OE*NRPS4* and Δ*NRPS4* with genomic DNA and negative controls all acquired 7 d after germination.

Conidia phenotype and germination

To examine whether deletion and overexpression of *NRPS4* induced other phenotypic changes of *Fusarium graminearum* we examined the sporulation of the three strains. When observed by light microscopy several cells of the OE*NRPS4* conidia appeared dark and empty or collapsed (Fig 4). This was observed for most of OE*NRPS4* conidia with the position and number of collapsed cells varying from conidia to conidia. The conidia of Δ*NRPS4* appeared similar to wild type under all observed conditions. Germination of conidia from the OE*NRPS4* and Δ*NRPS4* mutants were observed over a period of 24 h by light microscopy, including OE*NRPS4* conidia containing both empty and normal cells. Germination was observed for both OE*NRPS4* and Δ*NRPS4* conidia, but germination of OE*NRPS4* conidia was only observed from the normal cells and not from the collapsed areas (Fig 5).

To examine the spatial structures of the conidia we generated three-dimensional images by atomic force microscopy (AFM). With this technique we also observed empty cells in OE*NRPS4* conidia, which appear to be collapsed and flattened (Fig 6).

Examination of metabolite profiles

In order to identify the product of *NRPS4*, the wild type and mutant strains were grown on YES medium for two weeks before their metabolite profiles were examined by HPLC-UV and LC-MS. The results in Fig 7 show that the OE*NRPS4* mutant has a massive increase in production of the polyketide fusarin C and other fusarins compared to wild type. We were however not able to identify other secondary metabolites that were differentially produced by OE*NRPS4* compared to the wild type, although additional extractions with acetonitrile, heptane, hexane or methanol were attempted. Other methods such as heavy sonication or hydrophobin extraction protocols (Pedersen *et al.*, 2011) with trifluoroacetic acid along with boiling the tissue overnight in 0.1M - 1M NaOH and THF surfactant extraction (Yakimov *et al.*, 1995) also failed to extract a product that could be detected.

Discussion

The majority of fungal NRPS produced compounds have yet to be identified. The *NRPS4* gene has been found in a wide range of filamentous plant pathogenic fungi and the results here support previous reports that the product is involved in surface hydrophobicity and altered surface morphology of conidia.

The altered surface properties of the generated mutants suggested that the product of *NRPS4* could be a surfactant or involved in hydrophobin biosynthesis (Turgeon *et al.*, 2008). Different attempts at identifying the NRPS4 peptide proved unsuccessful. The results suggest that the NRP product could be embedded in the cell wall rather than being an excreted peptide. The extraction difficulties resemble those

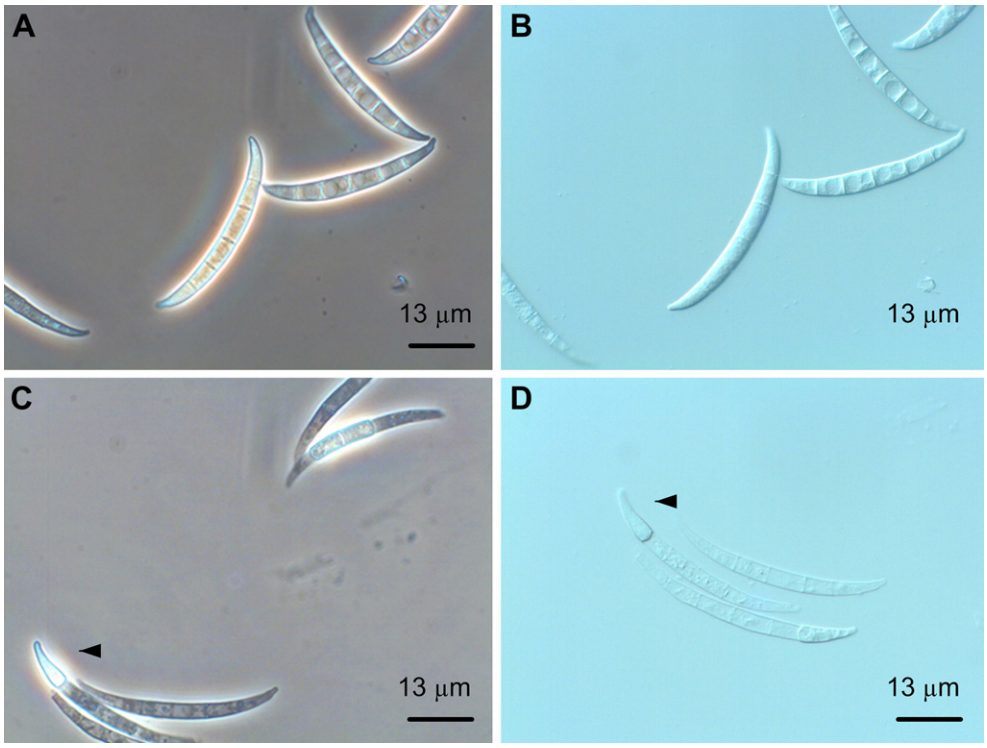

Fig 4 – Phase contrast microscopy (A, C). Differential interference microscopy (B, D). Conidia of the wild type PH1 (A, B). Conidia of the OE*NRPS4* mutant (B, D). The arrows indicate the bright and normal cells of the conidia from the OE*NRPS4* mutant. The remaining cells in these conidia remain dark and collapsed.

encountered in an attempt to locate and extract the NRP produced by *pes3* from *Aspergillus fumigatus* (O'Hanlon *et al.*, 2011). This six module NRPS is not a direct orthologue of *NRPS4*, but the *pes3* peptide is hypothesized to reside in the cell wall as it proved impossible to extract. The *pes3* deletion mutant had increased virulence and susceptibility to antifungal agents (O'Hanlon *et al.*, 2011). Deletion of the NRPS *pes1* from *A. fumigatus* resulted in altered surface morphology of conidia and decreased virulence and increased sensitivity to oxidative stress (Reeves *et al.*, 2006). This altered surface of conidia could indicate that the *pes1* peptide acts as a structural factor as may also be the case for the compound produced by *FgNRPS4*.

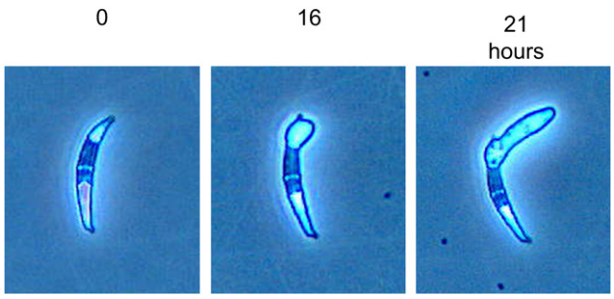

Fig 5 – Germination of a single conidia of OE*NRPS4* over a period of 21 hours. The time is denoted above the pictures. Germination is seen from the topmost cells.

Deletion of the *NRPS4* gene in *Cochloibolus heterostrophus* (*ChNRPS4*) has been found to generate an identical phenotype to *FgΔNRPS4* with a loss of surface hydrophobicity (Turgeon *et al.*, 2008). The *NRPS2* of *A. brassicicola* has also been investigated and a deletion of the gene resulted in a similar phenotype to the *FgΔNRPS4* mutant and the two genes share a 48.8 % consensus position identity (Kim *et al.*, 2007). A loss of surface hydrophobicity, an abnormal conidia wall structure and a loss of pathogenicity was observed in *AbΔNRPS2*, indicating that *AbNRPS2* might be an architectural factor of the cell wall. It will be interesting to examine if the deletion of *FgNRPS4* has similar effect on pathogenicity and explore if overexpression of the gene changes pathogenicity.

The conservation of *NRPS4* across the four sequenced *Fusarium* species and orthologues present in other filamentous fungi is remarkable. Apart from two of the siderophore synthetases, such a high degree of conservation is only seen for some of the smaller single module NRPSs (Hansen *et al.*, 2012). The modular structure suggests that the resulting peptide is five amino acids in length, although it could be longer as repetitive or iterative uses of modules in NRPSs have been observed (Haynes & Challis, 2007). Modules that can activate more than one specific amino acid substrate have also been reported (Bachmann & Ravel, 2009; Rausch *et al.*, 2005). Iterative modules are usually attributed to the siderophore synthetases and NRPSs that deviate from the normal domain order in their elongation modules (Mootz *et al.*, 2002). Using NRPSpredictor 2 (Rausch *et al.*, 2005; Röttig *et al.*, 2011), the nearest

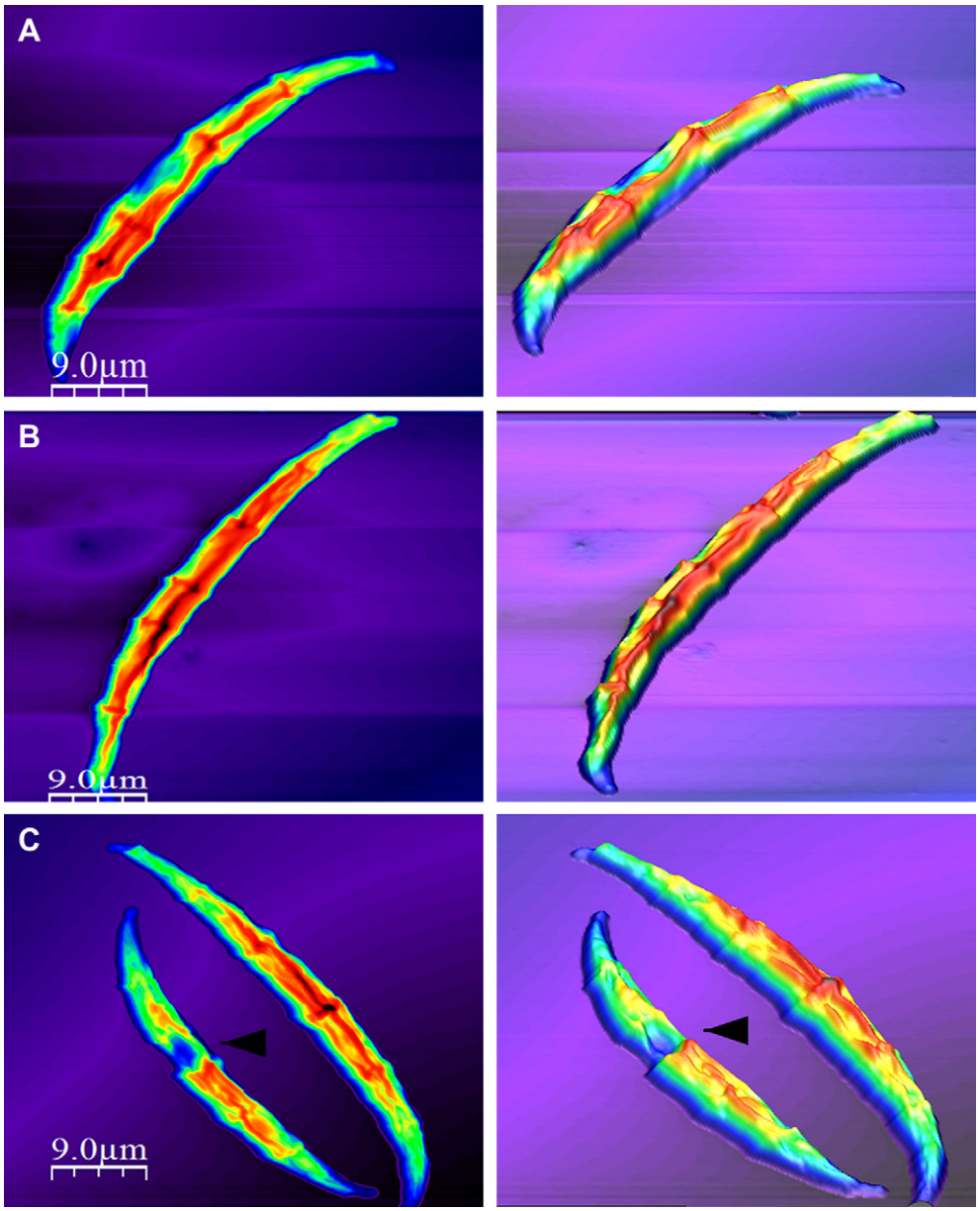

Fig 6 – Atomic force microscopy pictures of conidia from *F. graminearum* wild type and mutant strains. A) Wild type, B) ΔNRPS4, C) OENRPS4. The arrow indicate the flattened area of a collapsed cell.

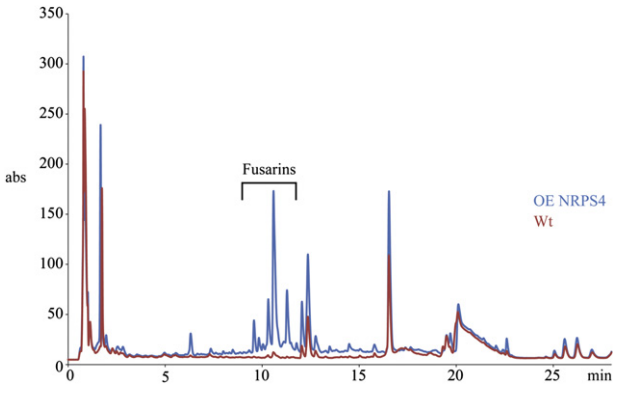

Fig 7 – HPLC-UV chromatogram of OENRPS4 (blue) and wild type (red) *F. graminearum* at 210 nm.

neighbor method and Stachelhaus code (Challis *et al.*, 2000; Stachelhaus *et al.*, 1999) the resulting pentapeptide is predicted to have the sequence cys-cys-gln-gln-ile with 50–60 % likelihood per substrate. *AbNRPS2* is orthologous to *ChNRPS4* and both have a 72.2 % residue identity as well as identical domain architecture. The domain architecture consists of a slightly reduced number of domains compared to *FgNRPS4* as the initial A domain is missing resulting in a slightly truncated gene. This is also indicated by a phylogenetic study of the adenylation (A) domains of an *Alternaria brassicae* NRPS, *AbNRPS2*, *ChNRPS4*, and *FgNRPS4* where all the respective A domains are clustered together (Kim *et al.*, 2007). Here the A domains of the first modules of the orthologues are clustered together with the second A domain of *FgNRPS4* and so forth. A neighboring putative ABC transporter found just downstream

of the *NRPS4* gene is conserved in all four sequenced *Fusarium* species (Hansen *et al*., 2012) and also appears to be present at a similar location to *AbNRPS2*, further linking the two genes. *Ab*Δ*NRPS2* also showed morphological differences in the conidia with an aberrant inflation of the cell wall and an increase in lipid bodies compared to the wild type (Kim *et al*., 2007).

The presence of the collapsed cells in the OE*NRPS4* mutant is curious as not all of the cells of each conidium are collapsed but only a few and at varying positions within the spore. This inconsistency could be explained by the fact that the *gpdA* promoter is affected by osmotic conditions resulting in heterogeneous effects on the cells. The orthologue *AbNRPS2* have been reported to be expressed almost exclusively in conidia and conidiophores, further indicating that *NRPS4* could play a role in conidial development (Kim *et al*., 2007). Another possibility could be that the NRPS4 peptide functions in a similar way to the cyclodepsipeptide beauvericin forming a cation ion selective channel across the cell membrane disrupting physiological conditions (Hamill *et al*., 1969; Kouri *et al*., 2003). This might also explain the increase in fusarin C production in the overexpression mutant as a lower water activity could be a consequence of the increased surface hydrophobicity of the mutant. Fusarin C has recently been purified from different fusarium species, and it is not likely that the compound is directly involved in hydrophobicity (Sondergaard *et al*., 2011). Water activity was shown to affect growth and secondary metabolite production in several studies (Aldred *et al*., 1999; Mogensen *et al*., 2009; Nielsen *et al*., 2004; Sepcic *et al*., 2011). In future works, it would be interesting to test the direct effect of fusarin C on surface hydrophobicity in wild type and *NRPS4* mutants.

This is the first time the *NRPS4* gene has been overexpressed and our results confirm that the gene is involved in hydrophobicity on cell surfaces. Like other studies of cell wall associated metabolites isolation of the compound was unsuccessful and further experiments are needed for identification of the NRPS4 product.

Acknowledgements

We would like to thank Dr. Jens Rafaelsen, Department of Physics and Nanotechnology, Aalborg University and Dr. Ole Højberg, Department of Animal Science, Aarhus University for help with microscopy. The research was supported by a grant from the Danish Agency for Science 10-100105.

Appendix A. Supplementary materials

Supplementary materials related to this article can be found online at doi:10.1016/j.funbio.2012.04.014

REFERENCES

Aldred D, Magan N, Lane BS, 1999. Influence of water activity and nutrients on growth and production of squalestatin S1 by a *Phoma* sp. *Journal of Applied Microbiology* **87**: 842–848.

Bachmann BO, Ravel J, 2009. *Methods For in Silico Prediction of Microbial Polyketide and Nonribosomal Peptide Biosynthetic Pathways From DNA Sequence Data*. Elsevier academic press inc, San Diego, CA, USA.

Bushley KE, Ripoll DR, Turgeon BG, 2008. Module evolution and substrate specificity of fungal nonribosomal peptide synthetases involved in siderophore biosynthesis. *BMC Evolutionary Biology* **8**: 328.

Calvo AM, Wilson RA, Bok JW, Keller NP, 2002. Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**: 447–459.

Challis GL, Ravel J, Townsend CA, 2000. Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. *Chemistry & Biology* **7**: 211–224.

Frandsen RJN, Andersson JA, Kristensen MB, Giese H, 2008. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. *BMC Molecular Biology* **9**: 70.

Gardiner DM, Kazan K, Manners JM, 2009. Novel genes of *Fusarium graminearum* that negatively regulate deoxynivalenol production and virulence. *Molecular Plant-Microbe Interactions* **22**: 1588–1600.

Hamill RL, Higgens CE, Boaz HE, Gorman M, 1969. Structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia Salina*. *Tetrahedron Letters* **49**: 4255–4258.

Hansen FT, Sørensen JL, Giese H, Sondergaard TE, Frandsen RJN, 2012. Quick guide to polyketide synthase and nonribosomal synthetase genes in *Fusarium*. *International Journal of Food Microbiology* **155**: 128–136.

Haynes SW, Challis GL, 2007. Non-linear enzymatic logic in natural product modular mega-synthases and -synthetases. *Current Opinion in Drug Discovery & Development* **10**: 203–218.

Kim KH, Cho Y, La Rota M, Cramer RA, Lawrence CB, 2007. Functional analysis of the *Alternaria brassicicola* non-ribosomal peptide synthetase gene *AbNPS2* reveals a role in conidial cell wall construction. *Molecular Plant Pathology* **8**: 23–39.

Kouri K, Lemmens M, Lemmens-Gruber R, 2003. Beauvericin-induced channels in ventricular myocytes and liposomes. *Biochimica et Biophysica Acta-Biomembranes* **1609**: 203–210.

Malz S, Grell MN, Thrane C, Maier FJ, Rosager P, Felk A, Albertsen KS, Salomon S, Bohn L, Schäfer W, Giese H, 2005. Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. *Fungal Genetics and Biology* **42**: 420–433.

Mogensen J, Nielsen K, Samson R, Frisvad J, Thrane U, 2009. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. *BMC Microbiology* **9**: 281.

Mootz HD, Schwarzer D, Marahiel MA, 2002. Ways of assembling complex natural products on modular nonribosomal peptide synthetases. *Chembiochem* **3**: 490–504.

Nielsen KF, Holm G, Uttrup LP, Nielsen PA, 2004. Mould growth on building materials under low water activities. Influence of humidity and temperature on fungal growth and secondary metabolism. *International Biodeterioration & Biodegradation* **54**: 325–336.

O'Hanlon KA, Cairns T, Stack D, Schrettl M, Bignell EM, Kavanagh K, Miggin SAM, O'Keeffe G, Larsen TO, Doyle S, 2011. Targeted disruption of nonribosomal peptide synthetase *pes3* augments the virulence of *Aspergillus fumigatus*. *Infection and Immunity* **79**: 3978–3992.

Pedersen M, Borodina I, Moresco J, Svendsen W, Frisvad J, Sondergaard I, 2011. High-yield production of hydrophobins RodA and RodB from *Aspergillus fumigatusin and Pichia pastoris*. *Applied Microbiology and Biotechnology* **90**: 1923–1932.

Rausch C, Weber T, Kohlbacher O, Wohlleben W, Huson DH, 2005. Specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPS) using transductive support vector machines (TSVMs). *Nucleic Acids Research* **33**: 5799–5808.

Reeves EP, Reiber K, Neville C, Scheibner O, Kavanagh K, Doyle S, 2006. A nonribosomal peptide synthetase (Pes1) confers protection against oxidative stress in *Aspergillus fumigatus*. *Febs Journal* **273**: 3038–3053.

Röttig M, Medema MH, Blin K, Weber T, Rausch C, Kohlbacher O, 2011. NRPSpredictor2-A web server for predicting NRPS adenylation domain specificity. *Nucleic Acids Research* **39**: 362–367.

Sepcic K, Zalar P, Gunde-Cimerman N, 2011. Low water activity induces the production of bioactive metabolites in halophilic and halotolerant fungi. *Marine Drugs* **9**: 43–58.

Smedsgaard J, 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *Journal of Chromatography A* **760**: 264–270.

Sondergaard TE, Hansen FT, Purup S, Nielsen AK, Giese H, Sørensen JL, 2011. FusarinC acts like an estrogenic agonist and stimulates breast cancer cells in vitro. *Toxicology Letters* **2**: 116–121.

Sørensen JL, Hansen FT, Sondergaard TE, Staerk D, Lee TV, Wimmer R, Purup S, Giese H, Frandsen RJN, 2012. Production of novel fusarielins by ectopic activation of the polyketide synthase 9 cluster in *Fusarium graminearum*. Environmental Microbiology, In press, DOI: 10.1111/j.1462-2920.2011.02696.x.

Stachelhaus T, Mootz HD, Marahiel MA, 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chemistry & Biology* **6**: 493–505.

Strieker M, Tanovic A, Marahiel MA, 2010. Nonribosomal peptide synthetases: structures and dynamics. *Current Opinion in Structural Biology* **20**: 234–240.

Tobiasen C, Aahman J, Ravnholt KS, Bjerrum MJ, Grell MN, Giese H, 2007. Nonribosomal peptide synthetase (NPS) genes in *Fusarium graminearum, F. culmorum* and *F. pseudograminearium* and identification of NPS2 as the producer of ferricrocin. *Current Genetics* **51**: 43–58.

Turgeon B, Oide S, Bushley K, 2008. Creating and screening *Cochliobolus heterostrophus* non-ribosomal peptide synthetase mutants. *Mycological Research* **112**: 200–206.

Varga J, Kocsube S, Toth B, Mesterhazy A, 2005. Nonribosomal peptide synthetase genes in the genome of *Fusarium graminearum*, causative agent of wheat head blight. *Acta Biologica Hungarica* **56**: 375–388.

von Döhren H, 2004. Biochemistry and general genetics of nonribosomal pepticle synthetases in fungi. *Molecular Biotechnology of Fungal Beta-Lactam Antibiotics and Related Peptide Synthetases* **88**: 217–264.

von Döhren H, Dieckmann R, Pavela-Vrancic M, 1999. The nonribosomal code. *Chemistry & Biology* **6**: R273–R279.

Yakimov MM, Timmis KN, Wray V, Fredrickson HL, 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* Bas50. *Applied and Environmental Microbiology* **61**: 1706–1713.